

# Hepatic stellate cell damage may lead to decreased plasma ADAMTS13 activity in rats

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**Abstract** ADAMTS13 is gaining attention, because its deficiency causes thrombotic thrombocytopenic purpura. Although its regulatory mechanism is not fully understood, we wondered if hepatic stellate cells (HSCs) play a role, because ADAMTS13 mRNA is exclusively expressed in the liver and primarily in HSCs. Plasma ADAMTS13 activity was markedly reduced in dimethylnitrosamine-treated rats, where HSC apoptosis is an essential event, but not in carbon tetrachloride- or thioacetamide-treated rats without HSC apoptosis. Furthermore, plasma ADAMTS13 activity was also reduced in 70% hepatectomized rats, where HSC loss occurs. These results suggest that HSC may be involved in the regulation of plasma ADAMTS13 activity.

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## 1. Introduction

A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) cleaves the multimers of von Willebrand factor (VWF) into smaller sizes [1]. VWF is synthesized as unusually large multimers of VWF (UL-VWF) in vascular endothelial cells [2]. After being released into plasma, UL-VWFs are rapidly cleaved by ADAMTS13 [1]. ADAMTS 13 is gaining attention, because it is unveiled that a defect in ADAMTS13 activity increases UL-VWFs in the plasma, causes platelet thrombosis under a high sheer stress, and finally results in Upshaw-Schulman syndrome by genetic mutations or thrombotic thrombocytopenic purpura (TTP) via inhibitory autoantibodies [3–5]; TTP is an important hematological illness characterized by thrombocytopenia, microangiopathic hemolytic anemia, fever, renal dysfunction, and central nervous system ischemia resulting from the formation of platelet thrombi within the microvasculature [3]. Vari-

ous levels of decreasing plasma ADAMTS13 activity were observed in other conditions such as hemolytic uremic syndrome [6], but the regulatory mechanism of this enzyme activity is not yet fully understood.

Recent studies revealed that ADAMTS13 mRNA is exclusively expressed in the liver, which may suggest that the liver is a major site involved in the synthesis of ADAMTS13 [7–9]. Moreover, it has been identified that ADAMTS13 mRNA is primarily expressed in hepatic stellate cells among the liver cells, suggesting that these cells are the major cells to synthesize ADAMTS13 [10,11]. Thus, we wondered whether damage to hepatic stellate cells might be one of the mechanisms of decreasing plasma ADAMTS13 activity.

Many chemical agents cause liver injury. Among them, we previously reported that dimethylnitrosamine (DMN) selectively causes the apoptosis of hepatic stellate cells in rats [12]. With this evidence, we wondered if plasma ADAMTS13 activity would be reduced in DMN-induced acute liver injury in rats, in comparison with carbon tetrachloride (CCl<sub>4</sub>)-induced acute liver injury in rats, where the apoptosis of hepatic stellate cells is not an essential event [12].

## 2. Materials and methods

**Animals.** Male Sprague–Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were fed a standard pelleted diet and water *ad libitum*, and used in all experiments. They were housed in groups of three or four per cage under normal laboratory conditions. All animals received human care in compliance with the Institution's Guidelines of the University of Tokyo and the National Institutes of Health Guidelines.

**Induction of acute liver injury by DMN, CCl<sub>4</sub> or thioacetamide (TAA) and plasma supplementation in rats.** Rats (weighing 170–190 g) received an intraperitoneal injection of 10, 25 or 50 mg/kg body weight of DMN as a 2.5% solution in saline and were sacrificed at various times after the injection up to 24 h [12]. In some DMN-treated rats, 2 ml of normal rat plasma was administered at 4 and 8 h after the DMN injection through inguinal vein, and rats were sacrificed at 12 h. To induce acute liver injury by CCl<sub>4</sub> or TAA, rats (weighing 170–190 g) received a subcutaneous injection of 2.0 mg/kg body weight of CCl<sub>4</sub> as a 20% solution in olive oil and were sacrificed at 24 h after the injection [12], or intraperitoneal injections of 300 mg/kg body weight of TAA in saline, twice at 24-h intervals, and were sacrificed at 48 h after the first injection [13].

**Blood collection and separation of plasma and serum.** Blood was collected through the inferior vena cava with a 21G needle and plastic syringe. Blood at 1.8 ml was mixed with 0.2 ml of 3.2% sodium citrate, and after measuring the platelet count, the rest of the citrated blood

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was centrifuged to separate the plasma. Serum was separated from non-citrated blood.

**Measurement of ADAMTS13 activity.** ADAMTS13 activity in the plasma was measured using an ADAMTS13 activity ELISA kit (Japan Clinical Laboratories Inc., Kyoto) [14].

**Measurement of platelet count and liver function.** The platelet count and serum levels of alanine aminotransferase (ALT) and albumin (Alb) were determined using an automated analyzer (Hitachi 7170; Hitachi Instruments Service Co., Ltd., Tokyo, Japan).

**Partial hepatectomy.** Partial hepatectomy (70%) was performed in rats according to the methods described by Higgins and Anderson [15]. Control rats underwent sham operations.

**Statistical analysis.** When indicated, statistical analysis was performed by Student's *t*-test, and *P* < 0.05 was considered significant.

### 3. Results and discussion

We previously demonstrated apoptosis of hepatic stellate cells occurs in rats with acute liver injury induced by DMN under electron microscopic observation, which is followed by sinusoidal endothelial cell degeneration and, thereafter, hepatocyte necrosis [12]. Fibrin thrombi appear in the sinusoids as well as in the necrotic area after the manifestation of hepatocyte necrosis [12]. In contrast, hepatocyte degeneration is firstly seen without hepatic stellate cell apoptosis and fibrin thrombi in the sinusoids of rats with acute liver injury induced by CCl<sub>4</sub> [12]. The fact that hepatic stellate cell apoptosis is not accompanied in CCl<sub>4</sub>-induced acute liver injury in rats was reported elsewhere [16–18]. Thus, DMN-induced acute liver injury is distinct from CCl<sub>4</sub>-induced injury in that hepatic stellate cells undergo apoptosis. In this study, we induced acute liver injury in rats with DMN or CCl<sub>4</sub>, as we previously described [12], and determined plasma ADAMTS13 activity in those rats. As demonstrated in Fig. 1A, plasma ADAMTS13 activity was markedly reduced in rats treated with 50 mg/kg body weight of DMN compared to untreated rats at 24 h after the injection. In contrast, plasma ADAMTS13 activity was not significantly altered in rats treated with 2.0 mg/kg body weight of CCl<sub>4</sub> compared to untreated rats at 24 h after the injection. On the other hand, ALT and albumin levels were not significantly different between DMN- and CCl<sub>4</sub>-treated rats, as shown in Table 1, indicating that there was no significant difference in hepatocyte damage between DMN and CCl<sub>4</sub> intoxication in rats. Furthermore, the reduction of plasma ADAMTS13 activity was dose-dependent in DMN-treated rats as depicted in Fig. 1B. These results suggest that the damage to hepatic stellate cells may explain the reduction of plasma ADAMTS13 activity in rats with acute liver injury induced by DMN.

To explore whether acute liver injury does not always cause reduced ADAMTS13 activity, it was determined in rats with TAA-induced liver injury [13], where apoptosis was reported in hepatocytes, but not in hepatic stellate cells [19]. As shown in Fig. 1A, plasma ADAMTS13 activity was not significantly altered in rats treated with 300 mg/kg body weight of TAA compared to untreated rats at 48 h after the injection. Serum ALT and albumin levels in TAA-treated rats were not significantly different from DMN- or CCl<sub>4</sub>-treated rats, as shown in Table 1. Thus, this result suggests that reduced ADAMTS13 activity may not be generally found in acute liver injury and the selective damage to hepatic stellate cells may be a key to the reduction of plasma ADAMTS13 activity in rats with acute liver injury.

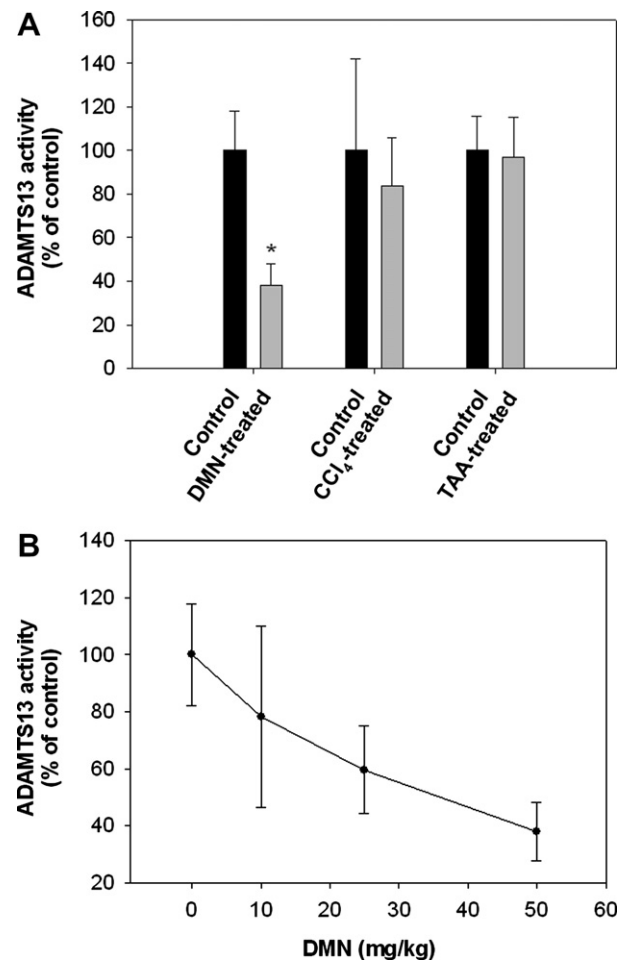


Fig. 1. (A) Plasma ADAMTS13 activity in DMN-, CCl<sub>4</sub>- or TAA-treated rats. Rats received an injection of 50 mg/kg body weight of DMN or an injection of 2.0 mg/kg body weight of CCl<sub>4</sub> and were sacrificed at 24 h after the injection, or intraperitoneal injections of 300 mg/kg body weight of TAA in saline, twice at 24-h intervals, and were sacrificed at 48 h after the first injection. ADAMTS13 activity was measured in the plasma using an ADAMTS13 activity ELISA kit. Columns and bars represent the means  $\pm$  S.D. of eight samples for DMN-treated rats and of four samples for CCl<sub>4</sub>- or TAA-treated rats. An asterisk indicates a significant difference between untreated control and DMN-treated rats (*P* < 0.05 or higher degree of significance). (B) Dose response of the effect of DMN on plasma ADAMTS13 activity in rats. Rats received an injection of 10, 25, 50 mg/kg body weight of DMN and were sacrificed at 24 h after the injection. ADAMTS13 activity was measured in the plasma as described above. Bars represent the mean  $\pm$  S.D. of eight samples.

Table 1  
Liver function and platelet count in rats treated with hepatotoxins

	ALT (IU/L)	Alb (g/dL)	Platelets ( $\times 10^4$ )
Control	39.2 $\pm$ 4.2	2.5 $\pm$ 0.0	102.2 $\pm$ 18.7
DMN-treated	1608.0 $\pm$ 993.4	2.5 $\pm$ 0.2	13.6 $\pm$ 7.5
CCl <sub>4</sub> -treated	674.5 $\pm$ 414.3	2.4 $\pm$ 0.1	85.6 $\pm$ 5.4
TAA-treated	1994.5 $\pm$ 836.2	2.3 $\pm$ 0.2	85.3 $\pm$ 18.4

Each value represents the mean  $\pm$  S.D. of eight samples for control or DMN-treated rats, and of four samples for CCl<sub>4</sub>-treated or TAA-treated rats.

Because our previous report showed that hepatic stellate cell apoptosis was first detected at 7.5 h and hepatocyte necrosis at

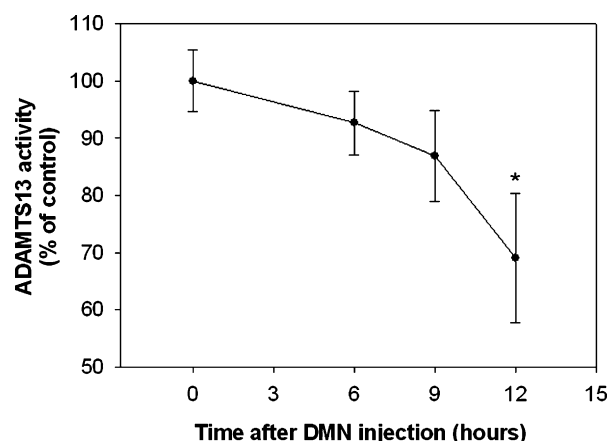


Fig. 2. Time-course analysis of plasma ADAMTS13 activity in DMN-treated rats. Rats received an injection of 50 mg/kg body weight of DMN and were sacrificed at 6, 9, and 12 h after the injection. ADAMTS13 activity in the plasma was measured using an ADAMTS13 activity ELISA kit. Bars represent the means  $\pm$  S.D. of six samples. An asterisk indicates a significant difference between untreated control and DMN-treated rats ( $P < 0.05$  or higher degree of significance).

12 h after DMN injection [12], we next performed a time-course analysis of plasma ADAMTS13 activity in DMN-induced liver injury. As depicted in Fig. 2, plasma ADAMTS13 activity was reduced in a time-dependent manner; a trend of reduction was noted at 6 h and a significant reduction was determined at 12 h after DMN injection. Thus, the timing of the reduction of plasma ADAMTS13 activity appeared to coincide with the initiation of hepatic stellate cell apoptosis. These results may be consistent with the hypothesis that hepatic stellate cell apoptosis would cause the reduction of plasma ADAMTS13 activity in DMN-induced liver injury.

As we previously reported, a markedly reduced peripheral platelet count was identified in rats treated with DMN (Table 1) [12,20]. This reduction was attributed to intravascular coagulation in the liver, because fibrin formation was noted in the hepatic sinusoids and antithrombin treatment inhibited platelet count reduction [12,20]. In this study, we raised the question of whether reduced plasma ADAMTS13 activity would play a role in platelet count reduction in DMN-induced liver injury. To answer this, DMN was injected in rats, and normal rat plasma at 2 ml was administered at 4 and 8 h and the platelet count and plasma ADAMTS13 activity were determined at 12 h after the injection. As shown in Fig. 3, an increase in plasma ADAMTS13 activity in DMN-treated rats with normal rat plasma administration was noted; however, there was a trend toward a decreasing platelet count in these rats. This result may suggest that the reduction of plasma ADAMTS13 activity did not play a role in the decrease of the peripheral platelet count in DMN-induced liver injury. It was reported that even when the ADAMTS13 gene was knocked out, thrombocytopenia was not found in mice, suggesting that intravascular thrombi are not necessarily caused by reduced plasma ADAMTS13 activity alone [21]. Our results may be in line with this report, and the relation between reduced plasma ADAMTS13 activity and thrombi production should be further elucidated.

Next, we employed partial hepatectomy in rats to examine whether the loss of hepatic stellate cells via surgery would lead

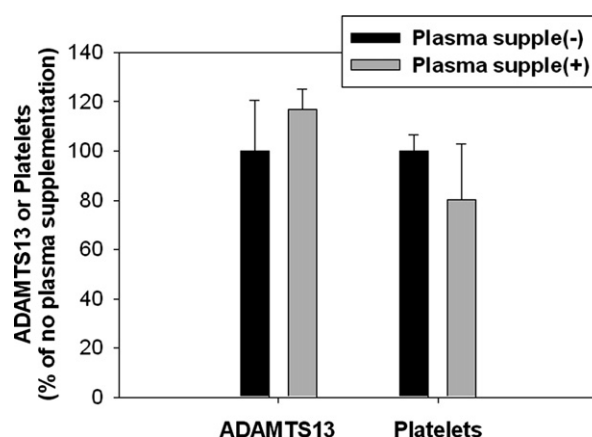


Fig. 3. Effect of normal rat plasma supplementation on plasma ADAMTS13 activity and platelet count in DMN-treated rats. Rats received an injection of 50 mg/kg body weight of DMN, and 2 ml of normal rat plasma was administered at 4 and 8 h after the injection. The plasma ADAMTS13 activity and platelet count were measured at 12 h after the injection. Columns and bars represent the means  $\pm$  S.D. of six samples.

to reduced plasma ADAMTS13 activity. Fig. 4 demonstrates that plasma ADAMTS13 activity was significantly reduced in rats with a 70% partial hepatectomy compared to sham-operated rats. A significant decrease in plasma ADAMTS13 activity was also reported in human cases with major hepatectomy [22].

Based on the evidence that hepatic stellate cells are important as cells which synthesize ADAMTS13 [10,11], we investigated the possibility that hepatic stellate cells play a role in determining plasma ADAMTS13 activity in this study. Then, we clarified that hepatic stellate cell damage via chemical toxin administration or hepatic stellate cell loss via surgery similarly causes a reduction in plasma ADAMTS13 activity. Recently, it was reported that human venous and arterial endothelial cells synthesize and release ADAMTS13, suggesting that the vast number of endothelial cells are another important source of

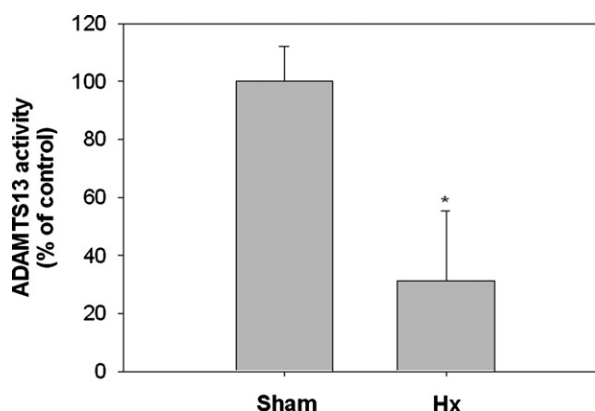


Fig. 4. Plasma ADAMTS13 activity in 70% hepatectomized rats. Rats underwent 70% hepatectomy (Hx) or sham operation (Sham) and were sacrificed at 24 h after the operation. ADAMTS13 activity was measured in the plasma using an ADAMTS13 activity ELISA kit. Columns and bars represent the means  $\pm$  S.D. of five samples. An asterisk indicates a significant difference between rats with a sham operation and 70% hepatectomy ( $P < 0.05$  or higher degree of significance).

ADAMTS13 [23,24]. However, in evaluating our current findings, it is unlikely that there would be a massive amount of damage caused to endothelial cells or vascular endothelium not only in the liver but also in the whole body in DMN-treated or hepatectomized rats, so the reduction of plasma ADAMTS13 activity is not attributable to endothelial cell or vascular endothelium damage in those rats.

It is now well known that hepatic stellate cells play a central role in the development of hepatic fibrosis [25]. In response to liver damage, hepatic stellate cells transdifferentiate to a myofibroblast-like phenotype with enhanced proliferation, fibrogenesis and contractility, in which process ADAMTS13 activity was shown to be increased in vitro and in vivo [26]. Of interest, ADAMTS13 activity was reported to be increased in the liver tissue lysate, but not in the plasma in rats with liver fibrosis induced by CCl<sub>4</sub> [26]. In patients with liver cirrhosis, ADAMTS13 activity in the plasma is shown to be decreased [27], and in contrast, highly variable [28]. Development of ADAMTS13 inhibitor was reported in a patient with hepatitis C virus-related liver cirrhosis [29]. These studies suggest that the regulatory mechanisms of plasma ADAMTS13 activity may be complicated in the chronic illness affecting the liver. However, our current evidence with animal experiments of acute liver injuries suggests that hepatic stellate cells may be involved at least in part in the regulation of plasma ADAMTS13 activity.

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